5   **Aventis Behring GmbH**   2002/M018 (A66)

Modified cDNA Factor VIII and its Derivatives

10

The present invention relates to modified DNA sequences coding for biologically active recombinant human factor VIII and its derivatives with improved stability, recombinant expression vectors containing such DNA sequences, host cells transformed with such recombinant expression vectors, and processes for the

15   manufacture of the recombinant human factor VIII and its derivatives. The invention also covers a transfer vector for use in human gene therapy which comprises such modified DNA sequences.

Classic hemophilia or hemophilia A is the most common of the inherited bleeding

20   disorders. It results from a chromosome X-linked deficiency of blood coagulation factor VIII, and affects almost exclusively males with an incidence of between one and two individuals per 10.000. The X-chromosome defect is transmitted by female carriers who are not themselves hemophiliacs. The clinical manifestation of hemophilia A is an abnormal bleeding tendency and before treatment with factor

25   VIII concentrates was introduced the mean life span for a person with severe hemophilia was less than 20 years. The use of concentrates of factor VIII from plasma has considerably improved the situation for the hemophilia patients. The mean life span has increased extensively, giving most of them the possibility to live a more or less normal life. However, there have been certain problems with the

30   plasma derived concentrates and their use, the most serious of which have been the transmission of viruses. So far, viruses causing AIDS, hepatitis B, and non A non B hepatitis have hit the population seriously. Although different virus inactivation methods and new highly purified factor VIII concentrates have recently been developed an inadvertant contamination can not be excluded. Also, the factor

5   VIII concentrates are fairly expensive because of the limited supply of human plasma raw material.

A factor VIII product derived from recombinant material is likely to solve a large extent of the problems associated with the use of plasma derived factor VIII

10   concentrates for treatment for hemophilia A. However, the development of a recombinant factor VIII has met some difficulties, for instance the problem of achieving production levels in sufficiently high yields, in particular regarding the full-length molecule.

15   In fresh plasma prepared in the presence of protease inhibitors, factor VIII has been shown to have a molecular weight of 280 kDa and to be composed of two polypeptide chains of 200 kDa and 80 kDa, respectively (Andersson, L.-O., et al. (1986) Proc. Natl. Acad. Sci. USA 83, 2979-2983). These chains are held together by metal ion bridges. More or less proteolytically degraded forms of the factor VIII

20   molecule can be found as active fragments in factor VIII material purified from commercial concentrates (Andersson, L.-O., et al. ibid.; Andersson, L.-O., et al. (1985) EP 0 197 901). The fragmented form of factor VIII having molecular weights from 260 kDa down to 170 kDa, consists of one heavy chain with a molecular weight ranging from 180 kDa down to 90 kDa, where all variants have identical

25   amino termini, in combination with one 80 kDa light chain. The amino-terminal region of the heavy chain is identical to that of the single chain factor VIII polypeptide that can be deduced from the nucleotide sequence data of the factor VIII cDNA (Wood, W.I., et al. (1984) Nature 312, 330-336; Vehar, G.A., et al. (1984) Nature 312, 337-342).

30

The smallest active form of factor VIII with a molecular weight of 170 kDa, consisting of one 90 kDa and one 80 kDa chain, can be activated with thrombin to the same extent as the higher molecular weight forms, and thus represents an unactivated form. It has also been shown to have full biological activity in vivo as

35   tested in hemophilia dogs (Brinkhous, K.M., et al. (1985) Proc. Natl. Acad. Sci. USA

5   82, 8752-8756). Thus, the haemostatic effectiveness of the 170 kDa form is the same as for the high molecular weight forms of factor VIII.

The fact that the middle heavily glycosylated region of the factor VIII polypeptide chain residing between amino acids Arg-740 and Glu-1649 does not seem to be
10   necessary for full biological activity has prompted several researchers to attempt to produce derivatives of recombinant factor VIII lacking this region. This has been achieved by deleting a portion of the cDNA encoding the middle heavily glycosylated region of factor VIII either entirely or partially.

15   For example, J.J. Toole, et al, reported the construction and expression of factor VIII lacking amino acids 982 through 1562, and 760 through 1639 respectively (Proc. Natl. Acad. Sci. USA (1986) 83, 5939-5942). D.L. Eaton, et al. reported the construction and expression of factor VIII lacking amino acids 797 through 1562 (Biochemistry (1986) 25, 8343-8347). R.J. Kaufman described the expression of
20   factor VIII lacking amino acids 741 through 1646 (PCT application No. WO 87/04187). N. Sarver, et al. reported the construction and expression of factor VIII lacking amino acids 747 through 1560 (DNA (1987) 6, 553-564). M. Pasek reported the construction and expression of factor VIII lacking amino acids 745 through 1562, and amino acids 741 through 1648, respectively (PCT application No. WO
25   88/00831). K.-D. Langner reported the construction and expression of factor VIII lacking amino acids 816 through 1598, and amino acids 741 through 1689, respectively (Behring Inst. Mitt., (1988) No. 82, 16-25, EP 0 295 597). P. Meulien, et al., reported the construction and expression of factor VIII lacking amino acids 868 through 1562, and amino acids 771 through 1666, respectively (Protein Engineering
30   (1988) 2(4), 301-306, EP 0 303 540). When expressing these deleted forms of factor VIII cDNA in mammalian cells the production level is typically 10 times higher as compared to full-length factor VIII.

FVIII is secreted into plasma as a heterodimer of a heavy chain (domains A1-A2-B)
35   and a light chain (A3-C1-C2) associated through a noncovalent divalent metal ion

5   linkage between the A1- and A3 domains. In plasma, FVIII is stabilized by binding to von Willebrand factor.

Upon proteolytic activation by thrombin, FVIII is activated to a heterotrimer of 2 heavy chain fragments (A1, a 50 kDa fragment, and A2 a 43 kDa fragment) and the

10   light chain (A3-C1-C2, a 73 kDa fragment). The active form of FVIII (FVIIIa) thus consists of an A1-subunit associated through the divalent metal ion linkage to a thrombin-cleaved A3-C1-C2 light chain and a free A2 subunit associated with the A1 domain . The dissociation of that free A2 subunit from the heterotrimer is thought to be the rate limiting step in FVIIIa inactivation after thrombin activation

15   (Fay, P.J. et al, J. Biol. Chem. 266: 8957 (1991), Fay PJ & Smudzin TM, J. Biol. Chem. 267: 13246-50 (1992)). The half life of FVIIIa in plasma is only 2.1 minutes (Saenko et al., Vox Sang. 83: 89-96 (2002)). To enhance the half life of FVIIIa would result into a longer acting FVIIIa which would also translate into less frequent injections of such a FVIII preparation. The inactivation of FVIIIa through

20   activated Protein C (APC) by cleavage at Arg336 and Arg562 is thought not to be the rate limiting step. Attempts have been made to create a FVIIIa which is inactivation resistant by covalently attaching the A2 domain to the A3 domain and by mutating the APC cleavage sites (Pipe and Kaufman, PNAS, 94:11851-11856). However such a FVIIIa could have a thrombogenic potential as it is almost

25   completely inactivation resistant. It is therefore the purpose of this invention to create a FVIIIa in which the A2 domain is stabilized without completely blocking inactivation.

FVIII is administered i.v. to haemophilia patients who are on prophylactic treatment

30   about 3 times per week due to the plasma half life of FVIII of about 12 hours. It would thus be highly desirable to create a FVIII with enhanced plasma half life which could lead to a FVIII preparation which has to be administered less frequently. The present invention offers a solution to this problem by a modified FVIII molecule with an increased association of A2 to the A1/A3-C1-C2.

35

5   The nature of these modifications was identified by comparing the sequence of porcine FVIII to that of human FVIII as it is known that the dissociation of human A2 domain is threefold enhanced versus that of porcine A2 (Lollar et al., J Biol. Chem., 267:23652-23657 (1992)). The sequence comparison (Fig. 1) revealed several differences. A subset of these differences consists of differently charged amino

10   acids. Mutants of human FVIII were constructed according to the following guidelines. When the human sequence contained a neutral amino acid whereas the porcine sequence contained a charged amino acid then a charged amino acid with the same charge as found in the porcine sequence was introduced into the human sequence. When the human sequence contained a charged amino acid whereas

15   the porcine FVIII contained a neutral amino acid then a neutral amino acid or an amino acid of the opposite charge was introduced, e.g. if the human FVIII contained an acidic amino acid at a position where the porcine FVIII contained a neutral amino acid, also a basic amino acid was introduced. When the human sequence contained a charged sequence whereas the porcine FVIII contained a charged

20   amino acid then an amino acid with the same charge as found in the porcine amino acid was introduced into the human sequence. Examples for such mutations which lead to an improved FVIII with a plasma half life of its activated form of more than three minutes, preferably of more than 10 minutes even more preferably more than 30 minutes, are listed in figure 2.

25

Other mutations for an improved FVIII were deduced by analyzing mutations in human FVIII which occurred naturally and which lead to a faster dissociation of the A2 domain associated with hemophilia. Such mutations result in differences between the two-stage assay as compared to the one-stage assay while

30   determining FVIII clotting activity, whereas the two-stage assay result is lower than that of the one-stage assay as in the two-stage assay an incubation time of several minutes allows an unstable A2 domain to dissociate (Saenko et al., Vox Sang., 83:89-96 (2002). It was inferred that in those cases where such an increased instability was the result of the introduction of a charged amino acid that amino acid

35   should be mutated into one of the opposite charge. Examples for such mutations

5   which lead to an improved FVIII with a plasma half life of its activated form of more than three minutes, preferably of more than 10 minutes, even more preferably more than 30 minutes, are listed in figure 3.

10   As a basis for introducing the mutations preferably a modified factor VIII cDNA is used which comprises a first DNA segment coding for the amino acids 1 through 740 of the human factor VIII and a second DNA segment coding for the amino acids 1649 through 2332 of the human factor VIII. These two segments may be interconnected by a linker DNA segment, but the invention also encompasses introducing the mutations into full length FVIII.

15

Subject of the invention is therefore a modified human factor VIII cDNA wherein mutations are inserted either in the wild-type factor VIII cDNA or in a factor VIII cDNA in which the B-domain is partially or completely deleted and may be replaced by a DNA linker segment, and

20

A)   one or several codons of the human factor VIII cDNA which are not identical with the corresponding codon in the same position of the porcine factor VIII cDNA are substituted by a different codon in such a way that

25   • when the human sequence contains a codon for a neutral amino acid whereas the porcine sequence contains a codon for a charged amino acid then a codon for an amino acid with the same charge as found in the porcine sequence is introduced into the human sequence;

30   • when the human sequence contains a codon for a charged amino acid whereas the porcine sequence contains a codon for a neutral amino acid then a codon for a neutral amino acid or a codon for an amino acid of the opposite charge is introduced into the human sequence

5 • when the human sequence contains a codon for a charged amino acid whereas the porcine sequence contains a codon for an amino acid with the opposite charge then a codon for an amino acid with the opposite charge is introduced into the human sequence or

10 B) one or several codons for a charged amino acid which are found in the FVIII cDNA of a hemophilic patient are replaced by a codon for an amino acid of the opposite charge.

The production of factor VIII proteins at high levels in suitable host cells, requires
15 the assembly of the above-mentioned modified factor VIII DNA's into efficient transcriptional units together with suitable regulatory elements in a recombinant expression vector, that can be propagated in E. coli according to methods known to those skilled in the art. Efficient transcriptional regulatory elements could be derived from viruses having animal cells as their natural hosts or from the chromosomal
20 DNA of animal cells. Preferably, promoter-enhancer combinations derived from the Simian Virus 40, adenovirus, BK polyoma virus, human cytomegalovirus, or the long terminal repeat of Rous sarcoma virus, or promoter-enhancer combinations including strongly constitutively transcribed genes in animal cells like beta-actin or GRP78 can be used. In order to achieve stable high levels of mRNA transcribed
25 from the factor VIII DNA's, the transcriptional unit should contain in its 3'-proximal part a DNA region encoding a transcriptional termination-polyadenylation sequence. Preferably, this sequence is derived from the Simian Virus 40 early transcriptional region, the rabbit beta-globin gene, or the human tissue plasminogen activator gene.

30

The factor VIII cDNA's are then integrated into the genome into a suitable host cell line for expression of the factor VIII proteins. Preferably this cell line should be an animal cell-line of vertebrate origin in order to ensure correct folding, disulfide bond formation, asparagines-linked glycosylation and other post-translational
35 modifications as well as secretion into the cultivation medium. Examples on other

5   post-translational modifications are tyrosine O-sulfation, and proteolytic processing of the nascent polypeptide chain. Examples of cell lines that can be use are monkey COS-cells, mouse L-cells, mouse C127-cells, hamster BHK-21 cells, human embryonic kidney 293 cells, and preferentially CHO-cells.

10   The recombinant expression vector encoding the factor VIII cDNA's can be introduced into an animal cell line in several different ways. For instance, recombinant expression vectors can be created from vectors based on different animal viruses, Examples of these are vectors based on baculovirus, vaccinia virus, adenovirus, and preferably bovine papilloma virus.

15

The transcription units encoding the factor VIII DNA's can also be introduced into animal cells together with another recombinant gene which may function as a dominant selectable marker in these cells in order to facilitate the isolation of specific cell clones which have integrated the recombinant DNA into their genome.

20   Examples of this type of dominant selectable marker genes are Tn5 aminoglycoside phosphotransferase, conferring resistance to Geneticin (G418), hygromycin phosphotransferase, conferring resistance to hygromycin, and puromycin acetyl transferase, conferring resistance to puromycin. The recombinant expression vector encoding such a selectable marker can reside either on the same vector as the one

25   encoding the factor VIII cDNA, or it can be encoded on a separate vector which is simultaneously introduced and integrated to the genome of the host cell, frequently resulting in a tight physical linkage between the different transcription units.

Other types of selectable marker genes which can be used together with the factor

30   VIII DNA's are based on various transcription units encoding dihydrofolate reductase (dhfr). After introduction of this type of gene into cells lacking endogenous dhfr-activity, preferentially CHO-cells (DUKX-B11, DG-44) it will enable these to grow in media lacking nucleosides. An example of such a medium is Ham's F12 without hypoxanthin, thymidin, and glycine. These dhfr-genes can be

35   introduced together with the factor VIII cDNA transcriptional units into CHO-cells of

5   the above type, either linked on the same vector or on different vectors, thus creating dhfr-positive cell lines producing recombinant factor VIII protein.

If the above cell lines are grown in the presence of the cytotoxic dhfr-inhibitor methotrexate, new cell lines resistant to methotrexate will emerge. These cell lines
10   may produce recombinant factor VIII protein at an increased rate due to the amplified number of linked dhfr and factor VIII transcriptional units. When propagating these cell lines in increasing concentrations of methotrexate (1-10000 nM), new cell lines can be obtained which produce factor VIII protein at very high rate.

15
The above cell lines producing factor VIII protein can be grown on a large scale, either in suspension culture or on various solid supports. Examples of these supports are microcarriers based on dextran or collagen matrices, or solid supports in the form of hollow fibres or various ceramic materials. When grown in cell
20   suspension culture or on microcarriers the culture of the above cell lines can be performed either as a bath culture or as a perfusion culture with continuous production of conditioned medium over extended periods of time. Thus, according to the present invention, the above cell lines are well suited for the development of an industrial process for the production of recombinant factor VIII that can be
25   isolated from human plasma.

The recombinant factor VIII protein which accumulate in the medium of CHO-cells of the above type, can be concentrated and purified by a variety of biochemical and chromatographic methods, including methods utilizing differences in size, charge,
30   hydrophobicity, solubility, specific affinity, etc. between the recombinant factor VIII protein and other substances in the cell cultivation medium.

An example of such a purification is the adsorption of the recombinant factor VIII protein to a monoclonal antibody which is immobilised on a solid support. After

5   desorption, the factor VIII protein can be further purified by a variety of chromatographic techniques based on the above properties.

The recombinant proteins with factor VIII activity described in this invention can be formulated into pharmaceutical preparations for therapeutic use. The purified factor

10   VIII proteins may be dissolved in conventional physiologically compatible aqueous buffer solutions to which there may be added, optionally, pharmaceutical adjuvants to provide pharmaceutical preparations.

The modified factor VIII DNA's of this invention may also be integrated into a

15   transfer vector for use in the human gene therapy.

A further subject of this invention is a modified biologically active recombinant human factor VIII with improved plasma half life of its activated form wherein mutations are inserted either in the wild-type factor VIII or in a FVIII in which the B-

20   domain is partially or completely deleted and replaced by a linker, and

A)   one or several amino acids of the human factor VIII which are not identical with the corresponding amino acid in the same position of the porcine factor VIII are substituted by a different amino acid in such a way that

25
- when the human sequence contains a neutral amino acid whereas the porcine sequence contains a charged amino acid then a charged amino acid with the same charge as found in the porcine sequence is introduced into the human sequence;

30
- when the human sequence contains a charged amino acid whereas the porcine sequence contains a neutral amino acid then a neutral amino acid or an amino acid of the opposite charge is introduced into the human sequence;

5   •   when the human sequence contains a charged amino acid whereas the porcine sequence contains an amino acid with the opposite charge then an amino acid with the opposite charge is introduced into the human sequence or

10   B)   one or several charged amino acids which are found in the FVIII amino sequence of hemophilic patients are replaced by a codon for an amino acid of the opposite charge.

The present invention will be further described more in detail in the following 15   examples thereof. This description of specific embodiments of the invention will be made in conjunction with the appended figures.

Generation of FVIII mutants

For the generation of FVIII mutants, a suitable subfragment of the FVIII cDNA (e.g. 20   Aval - Sacl, encompassing aminoacids 226 to 978) is first subcloned into a suitable cloning vector to reduce subsequent sequencing efforts. Site directed mutagenesis is then performed with a commercially available mutagenesis kit (e.g. QuickChange SiteDirected Mutagenesis Kit (Stratagene) according to the manufacturer´s instructions. Primers used for mutagenesis are listed in the attached sequence 25   listing and below, where the mutagenic bases are indicated in bold letters.

Mutation: A284K

Forward primer

$^{5´}$GGAACCATCGCCAG**AA**GTCCTTGGAAATCTCGCC$^{3´}$ (Sequence 1)

30   Reverse primer

$^{5´}$GGCGAGATTTCCAAGGAC**TT**CTGGCGATGGTTCC$^{3´}$ (Sequence 2)

Mutation: D318G

Forward primer

35   $^{5´}$CCCACCAACATG**G**TGGCATGGAAGCTTATGTC$^{3´}$ (Sequence 3)

5   Reverse primer
$5'$GACATAAGCTTCCATGCC**AC**CATGTTGGTGGG$^{3'}$ (Sequence 4)

Mutation: M337R

Forward   primer

10        $5'$CAGAGGAACCCCAACTACGA**CGT**AAAAATAATGAAGAAGCGGAAGAC$^{3'}$
            (Sequence 5)

Reverse   primer
            $5'$GTCTTCCGCTTCTTCATTATTTTT**ACG**TCGTAGTTGGGGTTCCTCTG$^{3'}$
            (Sequence 6)

15

Mutation: N340D

Forward   primer
            $5'$CCCAACTACGAATGAAAAT**G**ATGAAGAAGCGGAAGACTATG$^{3'}$
            (Sequence 7)

20 Reverse   primer
            $5'$CATAGTCTTCCGCTTCTTCAT**C**ATTTTTCATTCGTAGTTGGG$^{3'}$
            (Sequence 8)

Mutation: D349N

25 Forward   primer
            $5'$GAAGAAGCGGAAGACTATGATGAT**A**ATCTTACTGATTCTG$^{3'}$
            (Sequence 9)

Reverse primer $5'$CAGAATCAGTAAGAT**T**ATCATCATAGTCTTCCGCTTCTTC$^{3'}$
            (Sequence 10)

30

Mutation: N364D

Forward primer $5'$GGTCAGGTTTGATGATGAC**G**ACTCTCCTTCCTTTATCC$^{3'}$
            (Sequence 11)

Reverse primer $5'$GGATAAAGGAAGGAGAGT**C**GTCATCATCAAACCTGACC$^{3'}$

35   (Sequence 12)

5

Mutation: D403S

Forward primer ^{5}'CCCTTAGTCCTCGCCCCC**TC**TGACAGAAGTTATAAAAG$^{3'}$
  (Sequence 13)

Reverse primer ^{5}'CTTTTATAACTTCTGTCA**GA**GGGGGCGAGGACTAAGGG$^{3'}$

10   (Sequence 14)

Mutation: E434V

Forward   primer
    ^{5}'GTCCGATTTATGGCATACAGATG**TT**ACCTTTAAGACTCG$^{3'}$

15   (Sequence 15)

Reverse   primer
    ^{5}'CGAGTCTTAAAGGT**AA**CATCTGTGTATGCCATAAATCGGAC$^{3'}$
  (Sequence 16)

20

Mutation: E440K

Forward primer
^{5}'CCTTTAAGACTCGT**A**AAGCTATTCAGCATGAATCAGG$^{3'}$ (Sequence 17)

Reverse primer

25 ^{5}'CCTGATTCATGCTGAATAGCTT**T**ACGAGTCTTAAAGG$^{3'}$ (Sequence 18)

Mutation: Q468K

Forward   primer
    ^{5}'CACACTGTTGATTATATTTAAGAAT**A**AAGCAAGCAGACCATATAAC$^{3'}$

30   (Sequence 19)

Reverse   primer
    ^{5}'GTTATATGGTCTGCTTGCTT**T**ATTCTTAAATATAATCAACAGTGTG$^{3'}$
  (Sequence 20)

35   Mutation: R484S

5 Forward   primer

5'CCCTCACGGAATCACTGATGTC**TC**TCCTTTGTATTCAAGG³'

(Sequence 21)

Reverse   primer

5'CCTTGAATACAAAGGA**GA**GACATCAGTGATTCCGTGAGGG³'

10 (Sequence 22)

Mutation: R489G

Forward primer   5'GATGTCCGTCCTTTGTATTCA**G**GGAGATTACCAAAAGG³'

(Sequence 23)

15 Reverse primer   5'CCTTTTGGTAATCTCC**C**TGAATACAAAGGACGGACATC³'

(Sequence 24)

Mutation: . R583Q

Forward primer

20 5'CTGTATTTGATGAGAACC**A**AAGCTGGTACCTCACAG³' (Sequence 25)

Reverse primer

5'CTGTGAGGTACCAGCT**T**TGGTTCTCATCAAATACAG³' (Sequence 26)

Mutation: A599D

25 Forward primer

5'CTCCCCAATCCAG**A**TGGAGTGCAGCTTGAG³'   (Sequence 27)

Reverse primer

5'CTCAAGCTGCACTCCA**T**CTGGATTGGGGAG³'   (Sequence 28)

30 Mutation: E604Q

Forward primer

5'CAGCTGGAGTGCAGCTT**C**AGGATCCAGAGTTC³'   (Sequence 29)

Reverse primer

5'GAACTCTGGATCCT**G**AAGCTGCACTCCAGCTG³'   (Sequence 30)

35

5   Mutation: G1948K

Forward   primer

  $^{5'}$CGATGGTATCTGCTCAGCATG**AAG**AGCAATGAAAACATCCATTCTATT

C$^{3'}$ (Sequence 31)

Reverse   primer

10        $^{5'}$GAATAGAATGGATGTTTTCATTGCT**CTT**CATGCTGAGCAGATACCATCG

3'   (Sequence 32)

After clone isolation and sequence verification mutant subfragments are reinserted into the respective expression vector.

15

Expression of FVIII mutants

Transfection of FVIII mutant clones and expression of the mutant FVIII molecules is done as described previously and known to those skilled in the art (e.g. Plantier JL et al. Thromb. Haemost. 86:596-603 (2001)).

20

Measuring affinity of A2 subunit for A1/A3-C1-C2

The increased affinity of the A2 subunit for the A1/A3-C1-C2 can be measured as previously described by functional assays (Fay PJ & Smudzin TM. J. Biol. Chem. 267:13246-50 (1992); Lollar P et al. J. Biol. Chem. 267:23652-57 (1992)) as well as

25   a physical assay employing surface plasmon resonance (Persson E et al. Biochemistry 34:12775-81 (1995)).

The sequence of the porcine factor VIII is shown in Sequence 33, whereas the sequence of the human factor VIII is shown in sequence 34 of the attached

30   sequence listing.

In the following Sequence Listing Sequences 1-32 describe oligonucleotides which are used to introduce specific mutations into FVIII. Sequence 33 is the amino acid sequence of full length mature porcine FVIII, Sequence 34 is the amino acid

35   sequence of full length mature human FVIII.

SEQUENCE LISTING

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<140>   US/10/721,997
<141>   2003-11-26

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41

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41

<210> 17
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Human Factor VIII derivatives

<220>
<221> Forward primer
<222> (1)..(37)
<223>

<400> 17
cctttaagac tcgtaaagct attcagcatg aatcagg
37

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<211>  37
<212>  DNA
<213>  Artificial Sequence

<220>
<223>  Human Factor VIII derivatives

<220>
<221>  Reverse primer
<222>  (1)..(37)
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<400>  18
cctgattcat gctgaatagc tttacgagtc ttaaagg
37


<210>  19
<211>  46
<212>  DNA
<213>  Artificial Sequence

<220>
<223>  Human Factor VIII derivatives

<220>
<221>  Forward primer
<222>  (1)..(46)
<223>


<400>  19
cacactgttg attatattta agaataaagc aagcagacca tataac
46


<210>  20
<211>  46
<212>  DNA
<213>  Artificial Sequence

<220>
<223>  Human Factor VIII derivatives

<220>
<221>  Reverse primer
<222>  (1)..(46)
<223>


<400>  20
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gttatatggt ctgcttgctt tattcttaaa tataatcaac agtgtg
46

<210> 21
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Human Factor VIII derivatives

<220>
<221> Forward primer
<222> (1)..(40)
<223>

<400> 21
ccctcacgga atcactgatg tctctccttt gtattcaagg
40

<210> 22
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Human Factor VIII derivatives

<220>
<221> Reverse primer
<222> (1)..(40)
<223>

<400> 22
ccttgaatac aaaggagaga catcagtgat tccgtgaggg
40

<210> 23
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Human Factor VIII derivatives

<220>
<221> Forward primer
<222> (1)..(38)
<223>

```
<400>  23
gatgtccgtc ctttgtattc agggagatta ccaaaagg
38
```

```
<210>  24
<211>  38
<212>  DNA
<213>  Artificial Sequence

<220>
<223>  Human Factor VIII derivatives

<220>
<221>  Reverse primer
<222>  (1)..(38)
<223>
```

```
<400>  24
cctttttggta atctccctga atacaaagga cggacatc
38
```

```
<210>  25
<211>  36
<212>  DNA
<213>  Artificial Sequence

<220>
<223>  Human Factor VIII derivatives

<220>
<221>  Forward primer
<222>  (1)..(36)
<223>
```

```
<400>  25
ctgtatttga tgagaaccaa agctggtacc tcacag
36
```

```
<210>  26
<211>  36
<212>  DNA
<213>  Artificial Sequence

<220>
<223>  Human Factor VIII derivatives

<220>
<221>  Reverse primer
<222>  (1)..(36)
```

<223>

<400> 26
ctgtgaggta ccagctttgg ttctcatcaa atacag
36

<210> 27
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Human Factor VIII derivatives

<220>
<221> Forward primer
<222> (1)..(30)
<223>

<400> 27
ctccccaatc cagatggagt gcagcttgag
30

<210> 28
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Human Factor VIII derivatives

<220>
<221> Reverse primer
<222> (1)..(30)
<223>

<400> 28
ctcaagctgc actccatctg gattggggag
30

<210> 29
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Human Factor VIII derivatives

<220>

<221> Forward primer
<222> (1)..(32)
<223>

<400> 29
cagctggagt gcagcttcag gatccagagt tc
32

<210> 30
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Human Factor VIII derivatives

<220>
<221> Reverse primer
<222> (1)..(32)
<223>

<400> 30
gaactctgga tcctgaagct gcactccagc tg
32

<210> 31
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<213> Artificial Sequence

<220>
<223> Human Factor VIII derivatives

<220>
<221> Forward primer
<222> (1)..(49)
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<400> 31
cgatggtatc tgctcagcat gaagagcaat gaaaacatcc attctattc
49

<210> 32
<211> 49
<212> DNA
<213> Artificial Sequence

<220>
<223> Human Factor VIII derivatives

<220>
<221> Reverse primer
<222> (1)..(49)
<223>

<400> 32
gaatagaatg gatgttttca ttgctcttca tgctgagcag ataccatcg
49

<210> 33
<211> 2114
<212> PRT
<213> Porcine

<400> 33

Ala Ile Arg Arg Tyr Tyr Leu Gly Ala Val Glu Leu Ser Trp Asp Tyr
1   5   10   15

Arg Gln Ser Glu Leu Leu Arg Glu Leu His Val Asp Thr Arg Phe Pro
            20   25   30

Ala Thr Ala Pro Gly Ala Leu Pro Leu Gly Pro Ser Val Leu Tyr Lys
        35   40   45

Lys Thr Val Phe Val Glu Phe Thr Asp Gln Leu Phe Ser Val Ala Arg
    50   55   60

Pro Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln Ala Glu
65   70   75   80

Val Tyr Asp Thr Val Val Val Thr Leu Lys Asn Met Ala Ser His Pro
                85   90   95

Val Ser Leu His Ala Val Gly Val Ser Phe Trp Lys Ser Ser Glu Gly
            100   105   110

Ala Glu Tyr Glu Asp His Thr Ser Gln Arg Glu Lys Glu Asp Asp Lys
            115   120   125

Val Leu Pro Gly Lys Ser Gln Thr Tyr Val Trp Gln Val Leu Lys Glu
            130   135   140

```
Asn Gly Pro Thr Ala Ser Asp Pro Pro Cys Leu Thr Tyr Ser Tyr Leu
145             150             155                 160


Ser His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile Gly Ala
                165             170                 175


Leu Leu Val Cys Arg Glu Gly Ser Leu Thr Arg Glu Arg Thr Gln Asn
                180             185                 190


Leu His Glu Phe Val Leu Leu Phe Ala Val Phe Asp Glu Gly Lys Ser
            195             200             205


Trp His Ser Ala Arg Asn Asp Ser Trp Thr Arg Ala Met Asp Pro Ala
210                 215             220


Pro Ala Arg Ala Gln Pro Ala Met His Thr Val Asn Gly Tyr Val Asn
225                 230             235                 240


Arg Ser Leu Pro Gly Leu Ile Gly Cys His Lys Lys Ser Val Tyr Trp
            245             250                 255


His Val Ile Gly Met Gly Thr Ser Pro Glu Val His Ser Ile Phe Leu
            260             265                 270


Glu Gly His Thr Phe Leu Val Arg His His Arg Gln Ala Ser Leu Glu
        275             280             285


Ile Ser Pro Leu Thr Phe Leu Thr Ala Gln Thr Phe Leu Met Asp Leu
    290             295             300


Gly Gln Phe Leu Leu Phe Cys His Ile Ser Ser His His His Gly Gly
305             310             315                 320


Met Glu Ala His Val Arg Val Glu Ser Cys Ala Glu Glu Pro Gln Leu
            325             330                 335


Arg Arg Lys Ala Asp Glu Glu Glu Asp Tyr Asp Asp Asn Leu Tyr Asp
            340             345                 350


Ser Asp Met Asp Val Val Arg Leu Asp Gly Asp Asp Val Ser Pro Phe
            355             360             365
```

Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr Trp Val His
    370   375   380

Tyr Ile Ser Ala Glu Glu Glu Asp Trp Asp Tyr Ala Pro Ala Val Pro
385   390   395   400

Ser Pro Ser Asp Arg Ser Tyr Lys Ser Leu Tyr Leu Asn Ser Gly Pro
            405   410   415

Gln Arg Ile Gly Arg Lys Tyr Lys Lys Ala Arg Phe Val Ala Tyr Thr
          420   425   430

Asp Val Thr Phe Lys Thr Arg Lys Ala Ile Pro Tyr Glu Ser Gly Ile
        435   440   445

Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu Leu Ile Ile
    450   455   460

Phe Lys Asn Lys Ala Ser Arg Pro Tyr Asn Ile Tyr Pro His Gly Ile
465   470   475   480

Thr Asp Val Ser Ala Leu His Pro Gly Arg Leu Leu Lys Gly Trp Lys
          485   490   495

His Leu Lys Asp Met Pro Ile Leu Pro Gly Glu Thr Phe Lys Tyr Lys
        500   505   510

Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg Cys
        515   520   525

Leu Thr Arg Tyr Tyr Ser Ser Ser Ile Asn Leu Glu Lys Asp Leu Ala
        530   535   540

Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu Ser Val Asp
545   550   555   560

Gln Arg Gly Asn Gln Met Met Ser Asp Lys Arg Asn Val Ile Leu Phe
          565   570   575

Ser Val Phe Asp Glu Asn Gln Ser Trp Tyr Leu Ala Glu Asn Ile Gln
        580   585   590

Arg Phe Leu Pro Asn Pro Asp Gly Leu Gln Pro Gln Asp Pro Glu Phe
     595   600   605

Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val Phe Asp Ser
     610   615   620

Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp Tyr Ile Leu
625   630   635   640

Ser Val Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe Ser Gly Tyr
            645   650   655

Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr Leu Phe Pro
          660   665   670

Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro Gly Leu Trp
          675   680   685

Val Leu Gly Cys His Asn Ser Asp Leu Arg Asn Arg Gly Met Thr Ala
     690   695   700

Leu Leu Lys Val Tyr Ser Cys Asp Arg Asp Ile Gly Asp Tyr Tyr Asp
705   710   715   720

Asn Thr Tyr Glu Asp Ile Pro Gly Phe Leu Leu Ser Gly Lys Asn Val
            725   730   735

Ile Glu Pro Arg Ser Phe Ala Gln Asn Ser Arg Pro Pro Ser Ala Ser
          740   745   750

Gln Lys Gln Phe Gln Thr Ile Thr Ser Pro Glu Asp Asp Val Glu Leu
          755   760   765

Asp Pro Gln Ser Gly Glu Arg Thr Gln Ala Leu Glu Glu Leu Ser Val
     770   775   780

Pro Ser Gly Asp Gly Ser Met Leu Leu Gly Gln Asn Pro Ala Pro His
785   790   795   800

Gly Ser Ser Ser Ser Asp Leu Gln Glu Ala Arg Asn Glu Ala Asp Asp

```
                    805                      810                        815

Tyr Leu Pro Gly Ala Arg Glu Arg Asn Thr Ala Pro Ser Ala Ala Ala
            820                 825                 830

Arg Leu Arg Pro Glu Leu His His Ser Ala Glu Arg Val Leu Thr Pro
        835                 840                 845

Glu Pro Glu Lys Glu Leu Lys Lys Leu Asp Ser Lys Met Ser Ser Ser
    850                 855                 860

Ser Asp Leu Leu Lys Thr Ser Pro Thr Ile Pro Ser Asp Thr Leu Ser
865                 870                 875                     880

Ala Glu Thr Glu Arg Thr His Ser Leu Gly Pro Pro His Pro Gln Val
                885                 890                 895

Asn Phe Arg Ser Gln Leu Gly Ala Ile Val Leu Gly Lys Asn Ser Ser
            900                 905                 910

His Phe Ile Gly Ala Gly Val Pro Leu Gly Ser Thr Glu Glu Asp His
        915                 920                 925

Glu Ser Ser Leu Gly Glu Asn Val Ser Pro Val Glu Ser Asp Gly Ile
    930                 935                 940

Phe Glu Lys Glu Arg Ala His Gly Pro Ala Ser Leu Thr Lys Asp Asp
945                 950                 955                     960

Val Leu Phe Lys Val Asn Ile Ser Leu Val Lys Thr Asn Lys Ala Arg
            965                 970                 975

Val Tyr Leu Lys Thr Asn Arg Lys Ile His Ile Asp Asp Ala Ala Leu
            980                 985                 990

Leu Thr Glu Asn Arg Ala Ser Ala Thr Phe Met Asp Lys Asn Thr Thr
        995                 1000                1005

Ala Ser Gly Leu Asn His Val Ser Asn Trp Ile Lys Gly Pro Leu
    1010                1015                1020
```

```
Gly Lys Asn Pro Leu Ser Ser  Glu Arg Gly Pro Ser  Pro Glu Leu
    1025             1030             1035

Leu Thr Ser Ser Gly Ser Gly  Lys Ser Val Lys Gly  Gln Ser Ser
    1040             1045             1050

Gly Gln Gly Arg Ile Arg Val  Ala Val Glu Glu Glu  Glu Leu Ser
    1055             1060             1065

Lys Gly Lys Glu Met Met Leu  Pro Asn Ser Glu Leu  Thr Phe Leu
    1070             1075             1080

Thr Asn Ser Ala Asp Val Gln  Gly Asn Asp Thr His  Ser Gln Gly
    1085             1090             1095

Lys Lys Ser Arg Glu Glu Met  Glu Arg Arg Glu Lys  Leu Val Gln
    1100             1105             1110

Glu Lys Val Asp Leu Pro Gln  Val Tyr Thr Ala Thr  Gly Thr Lys
    1115             1120             1125

Asn Phe Leu Arg Asn Ile Phe  His Gln Ser Thr Glu  Pro Ser Val
    1130             1135             1140

Glu Gly Phe Asp Gly Gly Ser  His Ala Pro Val Pro  Gln Asp Ser
    1145             1150             1155

Arg Ser Leu Asn Asp Ser Ala  Glu Arg Ala Glu Thr  His Ile Ala
    1160             1165             1170

His Phe Ser Ala Ile Arg Glu  Glu Ala Pro Leu Glu  Ala Pro Gly
    1175             1180             1185

Asn Arg Thr Gly Pro Gly Pro  Arg Ser Ala Val Pro  Arg Arg Val
    1190             1195             1200

Lys Gln Ser Leu Lys Gln Ile  Arg Leu Pro Leu Glu  Glu Ile Lys
    1205             1210             1215

Pro Glu Arg Gly Val Val Leu  Asn Ala Thr Ser Thr  Arg Trp Ser
    1220             1225             1230
```

Glu Ser Ser Pro Ile Leu Gln Gly Ala Lys Arg Asn Asn Leu Ser
    1235   1240   1245

Leu Pro Phe Leu Thr Leu Glu Met Ala Gly Gly Gln Gly Lys Ile
    1250   1255   1260

Ser Ala Leu Gly Lys Ser Ala Ala Gly Pro Leu Ala Ser Gly Lys
    1265   1270   1275

Leu Glu Lys Ala Val Leu Ser Ser Ala Gly Leu Ser Glu Ala Ser
    1280   1285   1290

Gly Lys Ala Glu Phe Leu Pro Lys Val Arg Val His Arg Glu Asp
    1295   1300   1305

Leu Leu Pro Gln Lys Thr Ser Asn Val Ser Cys Ala His Gly Asp
    1310   1315   1320

Leu Gly Gln Glu Ile Phe Leu Gln Lys Thr Arg Gly Pro Val Asn
    1325   1330   1335

Leu Asn Lys Val Asn Arg Pro Gly Arg Thr Pro Ser Lys Leu Leu
    1340   1345   1350

Gly Pro Pro Met Pro Lys Glu Trp Glu Ser Leu Glu Lys Ser Pro
    1355   1360   1365

Lys Ser Thr Ala Leu Arg Thr Lys Asp Ile Ile Ser Leu Pro Leu
    1370   1375   1380

Asp Arg His Glu Ser Asn His Ser Ile Ala Ala Lys Asn Glu Gly
    1385   1390   1395

Gln Ala Glu Thr Gln Arg Glu Ala Ala Trp Thr Lys Gln Gly Gly
    1400   1405   1410

Pro Gly Arg Leu Cys Ala Pro Lys Pro Pro Val Leu Arg Arg His
    1415   1420   1425

Gln Arg Asp Ile Ser Leu Pro Thr Phe Gln Pro Glu Glu Asp Lys
    1430   1435   1440

Met Asp Tyr Asp Asp Ile Phe Ser Thr Glu Thr Lys Gly Glu Asp
    1445   1450   1455

Phe Asp Ile Tyr Gly Glu Asp Glu Asn Gln Asp Pro Arg Ser Phe
    1460   1465   1470

Gln Lys Arg Thr Arg His Tyr Phe Ile Ala Ala Val Glu Gln Leu
    1475   1480   1485

Trp Asp Tyr Gly Met Ser Glu Ser Pro Arg Ala Leu Arg Asn Arg
    1490   1495   1500

Ala Gln Asn Gly Glu Val Pro Arg Phe Lys Lys Val Val Phe Arg
    1505   1510   1515

Glu Phe Ala Asp Gly Ser Phe Thr Gln Pro Ser Tyr Arg Gly Glu
    1520   1525   1530

Leu Asn Lys His Leu Gly Leu Leu Gly Pro Tyr Ile Arg Ala Glu
    1535   1540   1545

Val Glu Asp Asn Ile Met Val Thr Phe Lys Asn Gln Ala Ser Arg
    1550   1555   1560

Pro Tyr Ser Phe Tyr Ser Ser Leu Ile Ser Tyr Pro Asp Asp Gln
    1565   1570   1575

Glu Gln Gly Ala Glu Pro Arg His Asn Phe Val Gln Pro Asn Glu
    1580   1585   1590

Thr Arg Thr Tyr Phe Trp Lys Val Gln His His Met Ala Pro Thr
    1595   1600   1605

Glu Asp Glu Phe Asp Cys Lys Ala Trp Ala Tyr Phe Ser Asp Val
    1610   1615   1620

Asp Leu Glu Lys Asp Val His Ser Gly Leu Ile Gly Pro Leu Leu
    1625   1630   1635

Ile Cys Arg Ala Asn Thr Leu Asn Ala Ala His Gly Arg Gln Val

```
           1640                    1645                      1650


Thr Val  Gln Glu Phe Ala Leu  Phe Phe Thr Ile Phe  Asp Glu Thr
         1655                  1660                 1665


Lys Ser  Trp Tyr Phe Thr Glu  Asn Val Glu Arg Asn  Cys Arg Ala
         1670                  1675                 1680


Pro Cys  His Leu Gln Met Glu  Asp Pro Thr Leu Lys  Glu Asn Tyr
         1685                  1690                 1695


Arg Phe  His Ala Ile Asn Gly  Tyr Val Met Asp Thr  Leu Pro Gly
         1700                  1705                 1710


Leu Val  Met Ala Gln Asn Gln  Arg Ile Arg Trp Tyr  Leu Leu Ser
         1715                  1720                 1725


Met Gly  Ser Asn Glu Asn Ile  His Ser Ile His Phe  Ser Gly His
         1730                  1735                 1740


Val Phe  Ser Val Arg Lys Lys  Glu Glu Tyr Lys Met  Ala Val Tyr
         1745                  1750                 1755


Asn Leu  Tyr Pro Gly Val Phe  Glu Thr Val Glu Met  Leu Pro Ser
         1760                  1765                 1770


Lys Val  Gly Ile Trp Arg Ile  Glu Cys Leu Ile Gly  Glu His Leu
         1775                  1780                 1785


Gln Ala  Gly Met Ser Thr Thr  Phe Leu Val Tyr Ser  Lys Glu Cys
         1790                  1795                 1800


Gln Ala  Pro Leu Gly Met Ala  Ser Gly Arg Ile Arg  Asp Phe Gln
         1805                  1810                 1815


Ile Thr  Ala Ser Gly Gln Tyr  Gly Gln Trp Ala Pro  Lys Leu Ala
         1820                  1825                 1830


Arg Leu  His Tyr Ser Gly Ser  Ile Asn Ala Trp Ser  Thr Lys Asp
         1835                  1840                 1845
```

```
Pro His  Ser Trp Ile Lys Val  Asp Leu Leu Ala Pro  Met Ile Ile
    1850             1855             1860

His Gly  Ile Met Thr Gln Gly  Ala Arg Gln Lys Phe  Ser Ser Leu
    1865             1870             1875

Tyr Ile  Ser Gln Phe Ile Ile  Met Tyr Ser Leu Asp  Gly Arg Asn
    1880             1885             1890

Trp Gln  Ser Tyr Arg Gly Asn  Ser Thr Gly Thr Leu  Met Val Phe
    1895             1900             1905

Phe Gly  Asn Val Asp Ala Ser  Gly Ile Lys His Asn  Ile Phe Asn
    1910             1915             1920

Pro Pro  Ile Val Ala Arg Tyr  Ile Arg Leu His Pro  Thr His Tyr
    1925             1930             1935

Ser Ile  Arg Ser Thr Leu Arg  Met Glu Leu Met Gly  Cys Asp Leu
    1940             1945             1950

Asn Ser  Cys Ser Met Pro Leu  Gly Met Gln Asn Lys  Ala Ile Ser
    1955             1960             1965

Asp Ser  Gln Ile Thr Ala Ser  Ser His Leu Ser Asn  Ile Phe Ala
    1970             1975             1980

Thr Trp  Ser Pro Ser Gln Ala  Arg Leu His Leu Gln  Gly Arg Thr
    1985             1990             1995

Asn Ala  Trp Arg Pro Arg Val  Ser Ser Ala Glu Glu  Trp Leu Gln
    2000             2005             2010

Val Asp  Leu Gln Lys Thr Val  Lys Val Thr Gly Ile  Thr Thr Gln
    2015             2020             2025

Gly Val  Lys Ser Leu Leu Ser  Ser Met Tyr Val Lys  Glu Phe Leu
    2030             2035             2040

Val Ser  Ser Ser Gln Asp Gly  Arg Arg Trp Thr Leu  Phe Leu Gln
    2045             2050             2055
```

Asp Gly His Thr Lys Val Phe Gln Gly Asn Gln Asp Ser Ser Thr
    2060   2065   2070

Pro Val Val Asn Ala Leu Asp Pro Pro Leu Phe Thr Arg Tyr Leu
    2075   2080   2085

Arg Ile His Pro Thr Ser Trp Ala Gln His Ile Ala Leu Arg Leu
    2090   2095   2100

Glu Val Leu Gly Cys Glu Ala Gln Asp Leu Tyr
    2105   2110

<210> 34
<211> 2332
<212> PRT
<213> Homo sapiens

<400> 34

Ala Thr Arg Arg Tyr Tyr Leu Gly Ala Val Glu Leu Ser Trp Asp Tyr
1   5   10   15

Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg Phe Pro Pro
            20   25   30

Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val Tyr Lys Lys
            35   40   45

Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile Ala Lys Pro
    50   55   60

Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln Ala Glu Val
65   70   75   80

Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser His Pro Val
                85   90   95

Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser Glu Gly Ala
            100   105   110

Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp Asp Lys Val
        115   120   125

Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu Lys Glu Asn
    130   135   140

Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser Tyr Leu Ser
145   150   155   160

His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile Gly Ala Leu
            165   170   175

Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu
            180   185   190

His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly Lys Ser Trp
        195   200   205

His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp Ala Ala Ser
    210   215   220

Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr Val Asn Arg
225   230   235   240

Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val Tyr Trp His
            245   250   255

Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile Phe Leu Glu
            260   265   270

Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser Leu Glu Ile
        275   280   285

Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met Asp Leu Gly
    290   295   300

Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His Asp Gly Met
305   310   315   320

Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro Gln Leu Arg
            325   330   335

Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp Leu Thr Asp
        340   345   350

Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser Pro Ser Phe
    355   360   365

Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr Trp Val His
    370   375   380

Tyr Ile Ala Ala Glu Glu Glu Asp Trp Asp Tyr Ala Pro Leu Val Leu
385   390   395   400

Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn Asn Gly Pro
          405   410   415

Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met Ala Tyr Thr
        420   425   430

Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu Ser Gly Ile
      435   440   445

Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu Leu Ile Ile
      450   455   460

Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro His Gly Ile
465   470   475   480

Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys Gly Val Lys
        485   490   495

His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe Lys Tyr Lys
        500   505   510

Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg Cys
      515   520   525

Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg Asp Leu Ala
      530   535   540

Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu Ser Val Asp
545   550   555   560

Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val Ile Leu Phe

                    565   570   575

Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu Asn Ile Gln
            580   585   590

Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp Pro Glu Phe
            595   600   605

Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val Phe Asp Ser
    610   615   620

Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp Tyr Ile Leu
625   630   635   640

Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe Ser Gly Tyr
            645   650   655

Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr Leu Phe Pro
            660   665   670

Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro Gly Leu Trp
            675   680   685

Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly Met Thr Ala
            690   695   700

Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp Tyr Tyr Glu
705   710   715   720

Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys Asn Asn Ala
            725   730   735

Ile Glu Pro Arg Ser Phe Ser Gln Asn Ser Arg His Arg Ser Thr Arg
            740   745   750

Gln Lys Gln Phe Asn Ala Thr Thr Ile Pro Glu Asn Asp Ile Glu Lys
            755   760   765

Thr Asp Pro Trp Phe Ala His Arg Thr Pro Met Pro Lys Ile Gln Asn
    770   775   780

Val Ser Ser Ser Asp Leu Leu Met Leu Leu Arg Gln Ser Pro Thr Pro
785   790   795   800

His Gly Leu Ser Leu Ser Asp Leu Gln Glu Ala Lys Tyr Glu Thr Phe
         805   810   815

Ser Asp Asp Pro Ser Pro Gly Ala Ile Asp Ser Asn Asn Ser Leu Ser
         820   825   830

Glu Met Thr His Phe Arg Pro Gln Leu His His Ser Gly Asp Met Val
         835   840   845

Phe Thr Pro Glu Ser Gly Leu Gln Leu Arg Leu Asn Glu Lys Leu Gly
    850   855   860

Thr Thr Ala Ala Thr Glu Leu Lys Lys Leu Asp Phe Lys Val Ser Ser
865   870   875   880

Thr Ser Asn Asn Leu Ile Ser Thr Ile Pro Ser Asp Asn Leu Ala Ala
             885   890   895

Gly Thr Asp Asn Thr Ser Ser Leu Gly Pro Pro Ser Met Pro Val His
         900   905   910

Tyr Asp Ser Gln Leu Asp Thr Thr Leu Phe Gly Lys Lys Ser Ser Pro
         915   920   925

Leu Thr Glu Ser Gly Gly Pro Leu Ser Leu Ser Glu Glu Asn Asn Asp
    930   935   940

Ser Lys Leu Leu Glu Ser Gly Leu Met Asn Ser Gln Glu Ser Ser Trp
945   950   955   960

Gly Lys Asn Val Ser Ser Thr Glu Ser Gly Arg Leu Phe Lys Gly Lys
             965   970   975

Arg Ala His Gly Pro Ala Leu Leu Thr Lys Asp Asn Ala Leu Phe Lys
             980   985   990

Val Ser Ile Ser Leu Leu Lys Thr Asn Lys Thr Ser Asn Asn Ser Ala
         995   1000   1005

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Thr Asn  Arg Lys Thr His Ile  Asp Gly Pro Ser Leu  Leu Ile Glu
    1010             1015             1020

Asn Ser  Pro Ser Val Trp Gln  Asn Ile Leu Glu Ser  Asp Thr Glu
    1025             1030             1035

Phe Lys  Lys Val Thr Pro Leu  Ile His Asp Arg Met  Leu Met Asp
    1040             1045             1050

Lys Asn  Ala Thr Ala Leu Arg  Leu Asn His Met Ser  Asn Lys Thr
    1055             1060             1065

Thr Ser  Ser Lys Asn Met Glu  Met Val Gln Gln Lys  Lys Glu Gly
    1070             1075             1080

Pro Ile  Pro Pro Asp Ala Gln  Asn Pro Asp Met Ser  Phe Phe Lys
    1085             1090             1095

Met Leu  Phe Leu Pro Glu Ser  Ala Arg Trp Ile Gln  Arg Thr His
    1100             1105             1110

Gly Lys  Asn Ser Leu Asn Ser  Gly Gln Gly Pro Ser  Pro Lys Gln
    1115             1120             1125

Leu Val  Ser Leu Gly Pro Glu  Lys Ser Val Glu Gly  Gln Asn Phe
    1130             1135             1140

Leu Ser  Glu Lys Asn Lys Val  Val Val Gly Lys Gly  Glu Phe Thr
    1145             1150             1155

Lys Asp  Val Gly Leu Lys Glu  Met Val Phe Pro Ser  Ser Arg Asn
    1160             1165             1170

Leu Phe  Leu Thr Asn Leu Asp  Asn Leu His Glu Asn  Asn Thr His
    1175             1180             1185

Asn Gln  Glu Lys Lys Ile Gln  Glu Glu Ile Glu Lys  Lys Glu Thr
    1190             1195             1200

Leu Ile  Gln Glu Asn Val Val  Leu Pro Gln Ile His  Thr Val Thr
    1205             1210             1215
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Gly Thr Lys Asn Phe Met Lys Asn Leu Phe Leu Leu Ser Thr Arg
    1220   1225   1230

Gln Asn Val Glu Gly Ser Tyr Asp Gly Ala Tyr Ala Pro Val Leu
    1235   1240   1245

Gln Asp Phe Arg Ser Leu Asn Asp Ser Thr Asn Arg Thr Lys Lys
    1250   1255   1260

His Thr Ala His Phe Ser Lys Lys Gly Glu Glu Glu Asn Leu Glu
    1265   1270   1275

Gly Leu Gly Asn Gln Thr Lys Gln Ile Val Glu Lys Tyr Ala Cys
    1280   1285   1290

Thr Thr Arg Ile Ser Pro Asn Thr Ser Gln Gln Asn Phe Val Thr
    1295   1300   1305

Gln Arg Ser Lys Arg Ala Leu Lys Gln Phe Arg Leu Pro Leu Glu
    1310   1315   1320

Glu Thr Glu Leu Glu Lys Arg Ile Ile Val Asp Asp Thr Ser Thr
    1325   1330   1335

Gln Trp Ser Lys Asn Met Lys His Leu Thr Pro Ser Thr Leu Thr
    1340   1345   1350

Gln Ile Asp Tyr Asn Glu Lys Glu Lys Gly Ala Ile Thr Gln Ser
    1355   1360   1365

Pro Leu Ser Asp Cys Leu Thr Arg Ser His Ser Ile Pro Gln Ala
    1370   1375   1380

Asn Arg Ser Pro Leu Pro Ile Ala Lys Val Ser Ser Phe Pro Ser
    1385   1390   1395

Ile Arg Pro Ile Tyr Leu Thr Arg Val Leu Phe Gln Asp Asn Ser
    1400   1405   1410

Ser His Leu Pro Ala Ala Ser Tyr Arg Lys Lys Asp Ser Gly Val

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        1415                    1420                    1425


Gln Glu  Ser Ser His Phe Leu  Gln Gly Ala Lys Lys  Asn Asn Leu
         1430                  1435                  1440


Ser Leu  Ala Ile Leu Thr Leu  Glu Met Thr Gly Asp  Gln Arg Glu
         1445                  1450                  1455


Val Gly  Ser Leu Gly Thr Ser  Ala Thr Asn Ser Val  Thr Tyr Lys
         1460                  1465                  1470


Lys Val  Glu Asn Thr Val Leu  Pro Lys Pro Asp Leu  Pro Lys Thr
         1475                  1480                  1485


Ser Gly  Lys Val Glu Leu Leu  Pro Lys Val His Ile  Tyr Gln Lys
         1490                  1495                  1500


Asp Leu  Phe Pro Thr Glu Thr  Ser Asn Gly Ser Pro  Gly His Leu
         1505                  1510                  1515


Asp Leu  Val Glu Gly Ser Leu  Leu Gln Gly Thr Glu  Gly Ala Ile
         1520                  1525                  1530


Lys Trp  Asn Glu Ala Asn Arg  Pro Gly Lys Val Pro  Phe Leu Arg
         1535                  1540                  1545


Val Ala  Thr Glu Ser Ser Ala  Lys Thr Pro Ser Lys  Leu Leu Asp
         1550                  1555                  1560


Pro Leu  Ala Trp Asp Asn His  Tyr Gly Thr Gln Ile  Pro Lys Glu
         1565                  1570                  1575


Glu Trp  Lys Ser Gln Glu Lys  Ser Pro Glu Lys Thr  Ala Phe Lys
         1580                  1585                  1590


Lys Lys  Asp Thr Ile Leu Ser  Leu Asn Ala Cys Glu  Ser Asn His
         1595                  1600                  1605


Ala Ile  Ala Ala Ile Asn Glu  Gly Gln Asn Lys Pro  Glu Ile Glu
         1610                  1615                  1620
```

Val Thr Trp Ala Lys Gln Gly Arg Thr Glu Arg Leu Cys Ser Gln
    1625   1630   1635

Asn Pro Pro Val Leu Lys Arg His Gln Arg Glu Ile Thr Arg Thr
    1640   1645   1650

Thr Leu Gln Ser Asp Gln Glu Glu Ile Asp Tyr Asp Asp Thr Ile
    1655   1660   1665

Ser Val Glu Met Lys Lys Glu Asp Phe Asp Ile Tyr Asp Glu Asp
    1670   1675   1680

Glu Asn Gln Ser Pro Arg Ser Phe Gln Lys Lys Thr Arg His Tyr
    1685   1690   1695

Phe Ile Ala Ala Val Glu Arg Leu Trp Asp Tyr Gly Met Ser Ser
    1700   1705   1710

Ser Pro His Val Leu Arg Asn Arg Ala Gln Ser Gly Ser Val Pro
    1715   1720   1725

Gln Phe Lys Lys Val Val Phe Gln Glu Phe Thr Asp Gly Ser Phe
    1730   1735   1740

Thr Gln Pro Leu Tyr Arg Gly Glu Leu Asn Glu His Leu Gly Leu
    1745   1750   1755

Leu Gly Pro Tyr Ile Arg Ala Glu Val Glu Asp Asn Ile Met Val
    1760   1765   1770

Thr Phe Arg Asn Gln Ala Ser Arg Pro Tyr Ser Phe Tyr Ser Ser
    1775   1780   1785

Leu Ile Ser Tyr Glu Glu Asp Gln Arg Gln Gly Ala Glu Pro Arg
    1790   1795   1800

Lys Asn Phe Val Lys Pro Asn Glu Thr Lys Thr Tyr Phe Trp Lys
    1805   1810   1815

Val Gln His His Met Ala Pro Thr Lys Asp Glu Phe Asp Cys Lys
    1820   1825   1830

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Ala Trp Ala Tyr Phe Ser Asp  Val Asp Leu Glu Lys  Asp Val His
    1835                1840              1845

Ser Gly Leu Ile Gly Pro Leu  Leu Val Cys His Thr  Asn Thr Leu
    1850                1855              1860

Asn Pro Ala His Gly Arg Gln  Val Thr Val Gln Glu  Phe Ala Leu
    1865                1870              1875

Phe Phe Thr Ile Phe Asp Glu  Thr Lys Ser Trp Tyr  Phe Thr Glu
    1880                1885              1890

Asn Met Glu Arg Asn Cys Arg  Ala Pro Cys Asn Ile  Gln Met Glu
    1895                1900              1905

Asp Pro Thr Phe Lys Glu Asn  Tyr Arg Phe His Ala  Ile Asn Gly
    1910                1915              1920

Tyr Ile Met Asp Thr Leu Pro  Gly Leu Val Met Ala  Gln Asp Gln
    1925                1930              1935

Arg Ile Arg Trp Tyr Leu Leu  Ser Met Gly Ser Asn  Glu Asn Ile
    1940                1945              1950

His Ser Ile His Phe Ser Gly  His Val Phe Thr Val  Arg Lys Lys
    1955                1960              1965

Glu Glu Tyr Lys Met Ala Leu  Tyr Asn Leu Tyr Pro  Gly Val Phe
    1970                1975              1980

Glu Thr Val Glu Met Leu Pro  Ser Lys Ala Gly Ile  Trp Arg Val
    1985                1990              1995

Glu Cys Leu Ile Gly Glu His  Leu His Ala Gly Met  Ser Thr Leu
    2000                2005              2010

Phe Leu Val Tyr Ser Asn Lys  Cys Gln Thr Pro Leu  Gly Met Ala
    2015                2020              2025

Ser Gly His Ile Arg Asp Phe  Gln Ile Thr Ala Ser  Gly Gln Tyr
    2030                2035              2040
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Gly Gln  Trp Ala Pro Lys Leu  Ala Arg Leu His Tyr  Ser Gly Ser
    2045              2050              2055

Ile Asn  Ala Trp Ser Thr Lys  Glu Pro Phe Ser Trp  Ile Lys Val
    2060              2065              2070

Asp Leu  Leu Ala Pro Met Ile  Ile His Gly Ile Lys  Thr Gln Gly
    2075              2080              2085

Ala Arg  Gln Lys Phe Ser Ser  Leu Tyr Ile Ser Gln  Phe Ile Ile
    2090              2095              2100

Met Tyr  Ser Leu Asp Gly Lys  Lys Trp Gln Thr Tyr  Arg Gly Asn
    2105              2110              2115

Ser Thr  Gly Thr Leu Met Val  Phe Phe Gly Asn Val  Asp Ser Ser
    2120              2125              2130

Gly Ile  Lys His Asn Ile Phe  Asn Pro Pro Ile Ile  Ala Arg Tyr
    2135              2140              2145

Ile Arg  Leu His Pro Thr His  Tyr Ser Ile Arg Ser  Thr Leu Arg
    2150              2155              2160

Met Glu  Leu Met Gly Cys Asp  Leu Asn Ser Cys Ser  Met Pro Leu
    2165              2170              2175

Gly Met  Glu Ser Lys Ala Ile  Ser Asp Ala Gln Ile  Thr Ala Ser
    2180              2185              2190

Ser Tyr  Phe Thr Asn Met Phe  Ala Thr Trp Ser Pro  Ser Lys Ala
    2195              2200              2205

Arg Leu  His Leu Gln Gly Arg  Ser Asn Ala Trp Arg  Pro Gln Val
    2210              2215              2220

Asn Asn  Pro Lys Glu Trp Leu  Gln Val Asp Phe Gln  Lys Thr Met
    2225              2230              2235

Lys Val  Thr Gly Val Thr Thr  Gln Gly Val Lys Ser  Leu Leu Thr
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        2240                    2245                        2250


Ser Met Tyr Val Lys Glu Phe Leu Ile Ser Ser Ser Gln Asp Gly
    2255                    2260            2265


His Gln Trp Thr Leu Phe Phe Gln Asn Gly Lys Val Lys Val Phe
    2270                    2275            2280


Gln Gly Asn Gln Asp Ser Phe Thr Pro Val Val Asn Ser Leu Asp
    2285                    2290            2295


Pro Pro Leu Leu Thr Arg Tyr Leu Arg Ile His Pro Gln Ser Trp
    2300                    2305            2310


Val His Gln Ile Ala Leu Arg Met Glu Val Leu Gly Cys Glu Ala
    2315                    2320            2325


Gln Asp Leu Tyr
    2330
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5   **Aventis Behring GmbH**   **2002/M018 (A66)**

Claims:

10

1.   Modified human factor VIII cDNA wherein mutations are inserted either in the wild-type factor VIII cDNA or in a factor VIII cDNA in which the B-domain is partially or completely deleted and may be replaced by a DNA linker segment, **characterised in that**

15

A)   one or several codons of the human factor VIII cDNA which are not identical with the corresponding codon in the same position of the porcine factor VIII cDNA are substituted by a different codon in such a way that

20   •   when the human sequence contains a codon for a neutral amino acid whereas the porcine sequence contains a codon for a charged amino acid then a codon for an amino acid with the same charge as found in the porcine sequence is introduced into the human sequence;

25

•   when the human sequence contains a codon for a charged amino acid whereas the porcine sequence contains a codon for a neutral amino acid then a codon for a neutral amino acid or a codon for an amino acid of the opposite charge is 30   introduced into the human sequence,

•   when the human sequence contains a codon for a charged amino acid whereas the porcine sequence contains a codon for an amino acid with the opposite charge then a codon for

5   an amino acid with the opposite charge is introduced into the human sequence or

B)   one or several codons for a charged amino acid which are found in the FVIII cDNA of a hemophilic patient are replaced by a codon for an amino

10   acid of the opposite charge.

2.   Recombinant expression vector containing the factor VIII cDNA as claimed in claim 1, **characterised in that** it carries in addition transcriptional regulatory elements for expression in a suitable host cell.

15

3.   Modified biologically active recombinant human factor VIII with improved stability wherein mutations are inserted either in the wild-type factor VIII or in a factor VIII in which the B-domain is partially or completely deleted and may be replaced by a linker, **characterised in that**

20

A.)   one or several amino acids of the human factor VIII which are not identical with the corresponding amino acid in the same position of the porcine factor VIII are substituted by a different amino acid in such a way that

25
- when the human sequence contains a neutral amino acid whereas the porcine sequence contains a charged amino acid then a charged amino acid with the same charge as found in the porcine sequence is introduced into the human sequence;

30
- when the human sequence contains a charged amino acid whereas the porcine sequence contains a neutral amino acid then a neutral amino acid or an amino acid of the opposite charge is introduced into the human sequence;

• when the human sequence contains a charged amino acid whereas the porcine sequence contains an amino acid with the opposite charge then an amino acid with the opposite charge is introduced into the human sequence or

B)   one or several charged amino acids which are found in the FVIII amino sequence of hemophilic patients are replaced by a codon for an amino acid of the opposite charge.

4.   Modified biologically active recombinant human factor VIII as claimed in claim 3, wherein the plasma half life of its activated form is more than 3 minutes, preferably more than 10 minutes and most preferably more than 30 minutes.

5.   Modified human factor VIII, **characterised in that** its A2-domain is stabilised by the substitution of one or several amino acids as claimed in claim 3.

6.   Process for the recombinant production of a modified human factor VIII as claimed in claim 3 either in cell suspension or on a solid support as a bath cell culture or as a perfusion cell culture with continuous production of a conditioned medium **characterised in that** the factor VIII proteins, which are expressed by a suitable host cell line are purified by chromatographic methods.

7.   Process as claimed in claim 6, **characterised in that** the transcription units encoding the modified factor VIII cDNA of claims 1 and 2 contain a dominant selectable marker in order to facilitate the isolation of specific cell clones which have integrated said specific c-DNA into their genome.

8.   Host cell line for expression of the factor VIII proteins of claim 3, **characterised in that** it is an animal cell line of vertebrate origin which contains the factor VIII cDNA of claim 1 integrated into its genome.

5 9.   Pharmaceutical composition, **characterised in that it** comprises a modified
biologically active recombinant human factor VIII of claim 3.

10.   Vector for gene therapy of hemophilia A, characterized in that it contains
a modified FVIII cDNA as claimed in claim 1.

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